

Remarks

Please cancel claims 4, 6, 16, 18 and 25-34.

Claims 1, 5, 7-8, 13-15, 17 and 19 are amended herein. Support for the amending language of claims 1-13 can be found in the specification on page 13, lines 13-34. Claim 1 is also amended to incorporate the limitations of claim 4 and to correct form. Claim 13 is also amended to incorporate the limitations of claim 16 and to correct form.

Claim 5 is amended to incorporate the limitations of claim 6. Claim 7 is amended to correct dependency. Support for the amendments of claim 8 can be found in the specification on page 7, lines 26-32. Claim 14 is amended to correct form. Claim 15 is amended to incorporate the limitations of claim 16. Claim 17 is amended to incorporate the limitations of claim 18. Claim 19 is amended to correct dependency.

New claims 35-40 are added herein. Following entry of this amendment, claims 1-3, 5, 7-15, 17, 19-24 and 35-40 are pending. Support for new claims 35-40 can be found throughout the specification, specifically on page 14, line 17 to page 15, line 6

Restriction Requirement

The restriction requirement has been made final. Thus, claims 25-34 are canceled herein as they were drawn to a non-elected invention.

Rejections Under 35 U.S.C. §112, first paragraph

Claims 1-24 are rejected as allegedly the specification does not provide sufficient written description for a sufficient number of species of polymorphisms that identify a preferred liver transplant donor. The Office action specifically asserts that the "specification fails to disclose additional polymorphisms in other regions [other than TNF- α] of the genomic DNA that is associated with an altered TNF activity can be used as a determinant for preferred liver donor." Applicant respectfully disagrees with this rejection as applied to the claims as amended.

The specification clearly provides a discussion of a representative number of species of genotypes of cytokines other than TNF- α that can identify a preferred liver transplant donor. Specifically, the specification identifies that, in addition to a polymorphism in TNF- α , a

polymorphic site is in a genomic nucleic acid encoding interleukin (IL)-1, IL-2, IL-6, IL-8, transforming growth factor (TGF)- β , IL-10, granulocyte macrophage colony stimulating factor (GMCSF), ciliary neurotrophic factor (CNTF) (see the specification at page 13, line 13 to page 13, line 34). Solely to advance prosecution, the claims 1 and 13 have now been amended to refer to polymorphisms in genomic sequences encoding (IL)-1, IL-2, IL-6, IL-8, transforming growth factor (TGF)- β , IL-10, granulocyte macrophage colony stimulating factor (GMCSF), ciliary neurotrophic factor (CNTF) of TNF- α . Thus, Applicant submits that the specification provides sufficient written description for claims 1 and 13 as amended, and claims that depend therefrom. Reconsideration and withdrawal of the rejection are respectfully requested.

With regard to new claims 35-40, Applicant notes that the Office action indicates that there is sufficient written description for a method of identifying a preferred liver donor utilizing a polymorphism at position -308 in the TNF- α promoter (see the Office action at page 3, paragraph 2). All of claims 35-40 specify that the polymorphism is at position -308 in the TNF- α promoter.

Claims 1-24 were rejected as allegedly the specification does not reasonably provide enablement for a method of identifying a preferred liver donor for transplantation by determining the presence of a preferred genotype, wherein the genotype is associated with an altered activity of TNF- α . Applicant respectfully disagrees with this assertion as applied to the claims as amended.

Claims 1 and 13 have been amended to recite that the polymorphic site is in a genomic nucleic acid sequence encoding interleukin (IL)-1, IL-2, IL-6, IL-8, transforming growth factor (TGF)- β , IL-10, granulocyte macrophage colony stimulating factor (GMCSF), ciliary neurotrophic factor (CNTF) or tumor necrosis factor (TNF- α). Applicant submits that the specification is fully enabling for methods for determining polymorphisms in the regulatory regions of these genes. These genes and preferred genotypes associated with the expression of these genes are described in the specification (see page 13, lines 13-34). For example, it is disclosed that a genotype at a polymorphic site that decreases activity of a cytokine, such as IL-10, that enhances TNF- α is a preferred genotype. In addition, it is disclosed that a genotype at a polymorphic site that is associated with activity of TGF- β is a preferred genotype. Methods

are also provided in the specification (see page 19, line 26 to page 25, line 3) for detecting specific polymorphisms. The specification further discloses an exemplary polymorphic site in TNF- α gene, TNF308.1, that is of use in the claimed methods.

Given the guidance provided by the specification, and the high level of knowledge of one of skill in the art, Applicant submits additional specific polymorphisms could readily be identified.

The ability to use additional polymorphisms is exemplified in a scientific manuscript published after the filing date of the present application, Tambur et al. (*Transplantation* 71:1475-1480, 2001, copy attached as Exhibit A). Tambur et al. describes an association between cytokine gene polymorphisms of TGF- β and IL-10 with the recurrence of hepatitis C in liver transplant recipients. As noted in Tambur et al., these allelic variations are known in the art and have been described in the scientific literature (see page 1475, and references 10-15 cited in Tambur et al., which are described as disclosing polymorphisms in cytokine genes¹). Applicant submits that the background section of Tambur et al. (see page 1475, column 2) demonstrates that those of skill in the art would be aware of these (and likely other) scientific publications describing the polymorphisms in cytokine genes such as TGF- β and IL-10.

The specification discloses that a genotype at a polymorphic site that is associated with activity of TGF- β is a preferred genotype (see the specification at page 13, line 27-29). Tambur et al. analyzed two previously known polymorphisms in TGF- β , one at position +10 (T versus C) and one at position +25 (G versus C) by PCR. A genetic profile of being able to produce high levels of TGF- β was apparent among patients resistant to disease recurrence (see Fig. 1 and page 1477, sentence bridging columns 1-2). This experimental data disclosed in Tambur et al. demonstrates that one of skill in the art, given the guidance provided by the specification, could readily use a TGF- β genotype to identify a preferred liver donor.

The specification further discloses that a genotype at a polymorphic site that decreases activity of a cytokine that enhances TNF- α , such as IL-10 is a preferred genotype (see the specification at page 13, lines 29-32). Tambur et al. analyzed three previously known polymorphisms in the IL-10 promoter, one at position -1082 (G versus A), one at position -819

¹ Fishman et al., *J. Clin. Invest.* 102:1369, 1998; He et al., *J. Neuroimmunol.* 86:13, 1998; Mulcahy et al., *Am. J. Hum. Genet.* 59:676, 1996; Cabrera et al., *J. Exp. Med.* 182:1259, 1995; Hohler et al., *Clin. Exp. Immunol.* 111:579, 1998; Hohler et al., *J. Med. Virol.* 54:173, 1998, all of which were published before the filing date of the present application.

(C versus T) and one at position -592 (A versus C) by PCR. The genetic profile of low production of IL-10 was also correlated with disease recurrence (see Fig. 2, page 1478, and page 1477, column 2, first full paragraph). Thus, the experimental data disclosed in Tambur et al. demonstrates that given the guidance provided by the specification, one of skill in the art could readily use an IL-10 genotype to identify a preferred liver donor.

The following analysis is submitted with regard to 1-3, 5, 7-15, 17, 19-24 (see the Office action at pages 6-7):

Nature of the invention

Methods are claimed for identifying a preferred liver donor using a polymorphic site in a genomic nucleic acid sequence encoding interleukin (IL)-1, IL-2, IL-6, IL-8, transforming growth factor (TGF)- β , IL-10, granulocyte macrophage colony stimulating factor (GMCSF), ciliary neurotrophic factor (CNTF) or tumor necrosis factor (TNF- α).

Breadth of the claim

The claims have been amended to be limited to the use of a polymorphic site in a genomic sequence encoding interleukin (IL)-1, IL-2, IL-6, IL-8, transforming growth factor (TGF)- β , IL-10, granulocyte macrophage colony stimulating factor (GMCSF), ciliary neurotrophic factor (CNTF) or tumor necrosis factor (TNF- α). A scientific manuscript published after the filing date of the present application, Tambur et al., supports the Applicant's assertion that the breadth of the claims is appropriate.

State of the Art and Skill Level of the Artisan

The art teaches a number of known polymorphisms in cytokine-genes. The art further teaches methods for identification of polymorphisms, such as PCR. Furthermore, the level of skill of a molecular biologist is high.

Amount of Experimentation

Using the techniques described in the above-referenced application, Tambur et al. obtained experimental data confirming the relationship of polymorphisms in IL-10 and TGF- β .

These results, obtained after the filing date of the present application, demonstrate that the amount of experimentation required was routine.

Thus, Applicant submits that claims 1-24 as amended are fully enabled by the specification. Reconsideration and withdrawal of the rejection are respectfully requested.

With regard to new claims 35-40, Applicant notes that the Office action states that the specification is enabling for identifying a preferred liver donor for transplantation by determining in the donor the presence of a polymorphism (a G) at nucleotide position -308 in the TNF- α promoter (see the Office action, page 4, paragraph 2).

Rejections Under 35 U.S.C. § 112, second paragraph

Claims 13-24 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly the word “material” is indefinite. Applicant respectfully disagrees with this assertion. However, solely to advance prosecution, the word “material” has been removed from the claims, rendering the rejection moot.

Claims 8-10 and 20-22 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly the term “TNF- α regulatory region” is indefinite. Applicant respectfully disagrees with this assertion.

The term “regulatory region” is clearly defined in the specification. Specifically, the specification at page 7, lines 26-32 states:

“As used herein, the term “regulatory region” refers to a portion of a nucleic acid molecule that determines the expression of a polypeptide encoded by a corresponding gene. A regulatory region that controls transcription of a gene can be a promoter, the site of initiation of transcription, or an enhancer, a DNA sequence that increases the rate of transcription....Regulatory regions include 5', 3' and intronic regulatory regions. The transcription regulatory regions control the transcription rate of the gene.” *[citations omitted]*

Thus, with regard to claims 8 and 20, the specification defines regulatory region as a nucleic acid sequence that regulates TNF- α expression. In addition, with regard to claims 9 and

21, the specification defines a "transcriptional regulatory region" as a nucleic acid sequence that controls the transcription rate (see the last sentence of the paragraph copied from the specification). Claims 10 and 21 depend from claims 9 and 20, respectively. Thus, Applicant submits that claims 8-10 and 20-22 are clear and definite. Reconsideration and withdrawal of the rejection are respectfully requested.

Prior Art

The Applicant thanks the Examiner for returning the signed copy of the Form PTO-1449, indicating that the references were considered and made of record. Applicant notes that no rejections were made of the cited prior art, indicating that the pending claims were free of the art of record.


Conclusion

It is submitted that pending claims 1-3, 5, 7-15, 17, 19-24 and 35-40 are in condition for allowance, which action is respectfully requested. The Applicant also thanks Examiner Qian for the brief introductory interview with the undersigned. If any matters remain to be discussed, it is requested that the Examiner contact the undersigned representative to arrange a telephone interview.

Respectfully submitted,

KLARQUIST SPARKMAN, LLP

By



Susan Alpert Siegel, Ph.D.
Registration No. 43,121

One World Trade Center, Suite 1600
121 S.W. Salmon Street
Portland, Oregon 97204
Telephone: (503) 226-7391
Facsimile: (503) 228-9446

ROLE OF CYTOKINE GENE POLYMORPHISM IN HEPATITIS C RECURRENCE AND ALLOGRAFT REJECTION AMONG LIVER TRANSPLANT RECIPIENTS¹

ANAT R. TAMBUR,^{2,3,6} JOHN W. ORTEGEL,² ZIV BEN-ARI,⁴ ETI SHABTAI,⁵ TIRZA KLEIN,⁶
RACHEL MICHOWIZ,⁷ RANI TUR-KASPA,⁴ AND EYTAN MOR⁷

Rush Medical Center, Chicago, Illinois; Rabin Medical Center, Petach-Tikva; and The Tel-Aviv University, Israel

Background. Cytokines play a key role in the regulation of immune responses. The maximal capacity of cytokine production varies between individuals and was shown to correlate with polymorphism in cytokine gene promoters. The objective of this study was to analyze the role of cytokine allelic variations in susceptibility to early graft rejection episodes and recurrence of hepatitis C infection in liver transplant (LTx) recipients.

Methods. The genetic profile of five cytokines was studied in 68 LTx recipients and 49 controls using polymerase chain reaction sequence specific primers. All individuals were genotyped as high or low producers of TNF- α and IL-6 and high, intermediate, or low producers of transforming growth factor β (TGF- β), interferon γ (IFN- γ), and interleukin 10 (IL-10) based on single nucleotide substitutions.

Results. No statistically significant differences were observed between patients with or without early rejection episodes. A significant proportion of patients more prone to rejection were genotyped as having a low production profile of IL-10 compared with the control population ($P=0.04$). These data are in accordance with reports regarding other solid-organ transplant recipients. Patients with no recurrence of hepatitis C had the inherent ability to produce higher TGF- β levels than did patients with recurrent disease ($P=0.042$). Among nonrecurrent patients, the percentage of genetically low IL-10 producers was higher than among recurrent patients ($P=0.07$). Furthermore, a genetic tendency to produce higher levels of IFN- γ was noted among LTx recipients with nonrecurrent hepatitis C than among those with recurrent hepatitis C.

Conclusions. While no significant correlation was detected between particular cytokine profile and early rejection episodes, our data strongly suggest an

association between cytokine gene polymorphism of TGF- β , IL-10, and IFN- γ and recurrence of hepatitis C in LTx recipients.

Modulation of immune responses, both cellular and humoral, is controlled primarily by secretion of cytokines from various cell types. These small peptides, in a complex coordinated network, can feed back onto their own synthesis or onto the induction/inhibition of other cytokines or cytokine receptors (1-4).

The in-vitro maximal capacity to produce different cytokines in response to mitogen stimulation was shown to vary between individuals. Such differences can be attributed to several molecular mechanisms, including variations in transcription, translation, and secretion pathways (5, 6). Recently, an additional potential mechanism was described involving conservative mutations within cytokines coding regions and nucleotide variations within more pronounced regulatory regions (i.e., promoter sequences). These genetic polymorphisms were shown to affect the overall expression and secretion of cytokines both in in-vitro and sporadically in in-vivo systems (7-9).

Potential association with allelic variations in certain cytokine genes was reported for several autoimmune syndromes (10-12), infectious diseases (13-15), and allograft rejection (16-19). In the transplantation setting, identification of a genetic marker predicting posttransplant complications may aid in tailoring immunosuppressive regimens to better suit individual patients' risk factors.

Two major immunological complications, acute rejection and recurrent disease, may lead to graft dysfunction in liver transplant (LTx) recipients. Acute cellular rejection, which usually occurs during the early posttransplant period (4-6 weeks), is noted in about 30-50% of patients. Recurrence of hepatitis C is more often a later consequence that may lead to graft failure. Although 95% of patients with hepatitis C remain viremic after transplant, the rate of symptomatic disease recurrence varies between those patients because of yet undefined reasons.

The objective of this study was to identify potential genetic markers for susceptibility to early graft rejection episodes and recurrence of hepatitis C in LTx recipients. To this end we analyzed genetic polymorphism in five different cytokines (tumor necrosis factor α [TNF- α], transforming growth factor β [TGF- β], interferon γ [IFN- γ], interleukin 6 [IL-6], and interleukin 10 [IL-10]). Allelic variations were compared between rejection and no-rejection groups and recurrence and

¹This work was presented in part at the First Joint Annual Meeting of the American Society for Transplant Surgeons and the American Society of Transplantation, May 13, 2000, Chicago, IL.

²Heart Failure and Cardiac Transplant Program, Rush Medical Center.

³Department of Immunology, Rush Medical College.

⁴The Liver Institute, Rabin Medical Center.

⁵The School of Health Professions, Sackler Faculty of Medicine, Tel-Aviv University.

⁶HLA laboratory, Rabin Medical Center.

⁷Department of Transplantation, Rabin Medical Center.

⁸Address correspondence to: Anat R Tambur, DMD, PhD, Department of Immunology, 1577 Jelke, Rush Medical College, 1653 W Congress Parkway, Chicago, IL 60612. e-mail: atambur@rush.edu.

no recurrence groups, and between those groups and healthy matched controls.

STUDY POPULATION AND METHODS

Patient population

Sixty-eight Caucasian LTx recipients (32 male, 36 female; age 20–69 years) followed in the Rabin Medical Center for 1 to 7 years posttransplantation were included in the study protocol after signing a consent form. Data from the patients' charts were reviewed for diagnosis of acute rejection (within the first 6 weeks after transplant) and recurrent hepatitis C (within the first year after transplant). Diagnosis of recurrent hepatitis C was based on viremia [by reverse transcriptase-polymerase chain reaction (RT-PCR)], liver enzyme abnormalities, and typical histological characteristics on biopsy. The reason for end-stage liver disease among the 68 patients included: hepatitis C [17], hepatitis B [16], hepatitis B and C [13], Wilson's disease [4], primary biliary cirrhosis [8], primary sclerosing cholangitis [2], fulminant hepatic failure [9], cryptogenic cirrhosis [5], and other etiologies [4]. Immunosuppression therapy consisted of CsA, azathioprine, plus steroids in 29 patients and tacrolimus (FK 506) plus steroids in the remaining 39 patients.

Controls

Forty-nine healthy Caucasian individuals were analyzed as a representative control population: 23 male, 26 female; age 18–64 years.

DNA Extraction

Genomic DNA was isolated by proteinase K digestion of fresh peripheral blood mononuclear cells, followed by phenol extraction and ethanol precipitation. DNA samples were quantified and subjected to specific PCR reactions as described.

Cytokine Gene Polymorphism

Single nucleotide mutations were analyzed in five different cytokines, leading to genotypes and phenotypes assignment. Specifically, for TNF- α , the presence of G or A nucleotide in position -308 of the promoter region generates three potential genotypes corresponding to two different phenotypes. The A/A and G/A genotypes represent the potential to produce high levels of TNF- α , whereas the G/G genotype is associated with low production of this proinflammatory cytokine. Two single nucleotide mutations were analyzed for TGF- β , both in the coding region: codon +10 can be either T or C and codon +25 either C or G. There are 9 different possible combinations of these two isolated mutations, which gives rise to three different secretion phenotypes: high, intermediate, and low producers of TGF- β . An additional coding sequence mutation was analyzed for IFN- γ at position +874 (T versus A). A homozygous T is associated

with the ability to produce high levels of IFN- γ , the heterozygous T/A is an intermediate producer, and the homozygous A genotype can generate only low amounts of IFN- γ . Interleukin-6 promoter was studied for the presence of a single nucleotide modification in position -174. Both the G/G and the G/C genotypes are known to correlate with a high production phenotype, whereas the C/C is a low producer of IL-6.

Three different polymorphisms were surveyed for the IL-10 promoter: position -1082 (G versus A), position -819 (C versus T), and position -592 (A versus C). The phenotypes attributed to these polymorphisms are presented in Table 2.

PCR-SSP

PCR amplification was carried out according to manufacturer's recommendations (One Lambda, Inc. Canoga park, CA). Briefly, after the addition of the appropriate primer pairs, salts, buffer, and Taq polymerase, the samples were subjected to 30 cycles of PCR as follows: One cycle of 130 seconds at 96°C dropping to 63°C for an additional 60 seconds; 9 cycles of 10 seconds at 96°C and 60 seconds at 63°C; and the final 20 cycles that include a three-temperature ramp—annealing for 10 seconds at 96°C, hybridization for 50 seconds at 59°C and an extension step of 30 seconds at 72°C. PCR products were then loaded onto an agarose gel and photographed using an ultraviolet transilluminator.

Statistical analysis

Comparison of cytokine profiles among the different patient groups and between patients and control population were performed using Chi-square analysis and two-tailed Fisher's exact test, as appropriate.

RESULTS

Rejection

of the 63 recipients for whom information regarding early rejection episodes was available, 33 (52%) presented with at least one biopsy-documented rejection and 30 had no rejection events. Demographic characteristics (i.e., age, sex) and immunosuppression protocols of patients with and without rejection were comparable.

Table 1 summarizes the phenotypic expression deduced from the genetic polymorphism in the five selected cytokines in the two patient groups and the data obtained for healthy controls. Overall, no statistically significant differences were observed between patients susceptible for early rejection episodes and patients that did not present with rejections. Frequencies of cytokine gene polymorphism in both patient

TABLE 1. Phenotypic expression of the selected five cytokines in LTx recipients with and without rejection episodes within the first 6 weeks posttransplant and in healthy controls

within the first 6 weeks posttransplant and in healthy controls							
	TNF- α		IL-6		IFN- γ		
	Low	High	Low	High	Low	Intermediate	High
Rejection	30 (91)	3 (9)	1 (3)	32 (97)	11 (34)	17 (50)	5 (16)
No rejection	26 (87)	4 (13)	4 (13) ^a	26 (87)	11 (35)	13 (46)	6 (19)
Control	43 (88)	6 (12)	1 (2) ^a	48 (98)	19 (39)	24 (49)	6 (12)
	TGF- β			IL-10			
	Low	Intermediate	High	Low	Intermediate	High	
Rejection	1 (3)	8 (22)	24 (75)	19 (56) ^b	10 (32)	4 (12)	
No rejection	2 (6)	12 (42)	16 (52)	14 (48)	9 (29)	7 (23)	
Control	3 (6)	10 (20)	36 (73)	17 (35) ^b	26 (53)	6 (12)	

Numbers in parenthesis are %.

^a $P=0.066$ for low IL-6 between patients with no rejection and the control group.

^b $P=0.041$ for low IL-10 between patients with rejection and the control group.

TABLE 2. Frequency of IL-10 genotypes observed in LTx recipients with and without rejection episodes in the early posttransplant period and in healthy controls

Nucleotide combination	Interleukin-10 level of production					
	High	Intermediate		Low		
	GCC/GCC	GCC/ACC	GCC/ATA	ACC/ACC	ACC/ATA	ATA/ATA
Rejection	4 (12)	5 (15)	5 (15)	3 (9)	11 (33)	5 (15)
No rejection	7 (23)	5 (17)	4 (13)	7 (23)	6 (20)	1 (3)
Healthy controls	6 (12)	13 (27)	13 (27)	4 (8)	8 (16)	5 (10)

The letters in each nucleotide-triplet represent a combination of all three polymorphic regions, i.e., positions -1082, -819, and -592. For example, GCC corresponds to G in position -1082, C in position -819, and C in position -592. An individual who is homozygous for this polymorphism will have a high IL-10 production phenotype. Numbers in parenthesis are %.

populations were also similar to those noted in the control group.

The majority of patients and controls (85–90%) had a low TNF- α phenotype—G/G. Of the few individuals typed as high producers, all were heterozygous for the polymorphism at position -308 (i.e., G/A). None of the patients or controls carried the A/A homozygous high-producer type (data not shown).

The vast majority (87–98%) of all populations studied exhibit the potential to produce high levels of IL-6, using either the G/G or the G/C genotypes equally. The low-production genotype (C/C) was more frequent among the nonrejection group, showing a trend toward statistical significance when compared with the control population ($P=0.066$).

The allelic polymorphisms of IFN- γ translate directly into the three phenotypic levels of expression. Consequently, no differences were noted with regard to either the phenotype or the genotype profile of patients with or without rejection episodes.

TGF- β genotypes varied quite substantially among the different study groups (data not shown). In LTx recipients with no rejection, the two high production profiles were equally distributed, whereas in both LTx recipients with rejection episodes and the control population the more-frequent high-producer haplotype was T/C G/G. The most prevalent genotype among all patients with no rejection episodes was the C/C G/G (intermediate) haplotype; 11/30. These differences did not reach statistical significance.

Analyzing the different genotypes of IL-10: a trend toward higher frequency of high IL-10 producers was found in the non-rejection LTx recipients (23% versus 12% in the rejection group) (Table 2). Although higher frequencies of the low producing genotypes - ACC/ATA and ATA/ATA - were found in the rejection group (33% and 15% versus 20% and 3%, respectively, in the no-rejection set), a similar proportion of low IL-10 producers was observed among these two groups overall (57% versus 46%). Nonetheless, when the secretion profiles were pooled into two categories - high and intermediate producers versus low producers - a significantly higher percent of patients in the rejection group showed reduced genetic ability to produce IL-10 (low producers) compared with control population ($P=0.041$; NS - compared with no-rejection).

Hepatitis C

Among the 20 LTx recipients with primary hepatitis C, 12 had documented disease recurrence within the first year post transplant. A distinct ability to produce high levels of TGF- β

TGF- β Genetic Profile

LTx Recipients with primary Hepatitis C

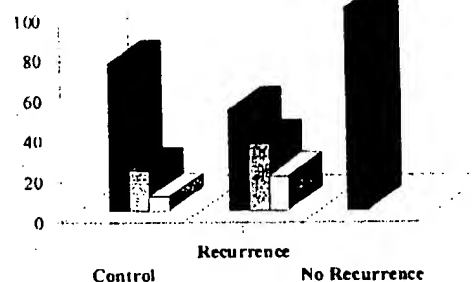


FIGURE 1. TGF- β profile in LTx recipients with primary hepatitis C. The three study groups include patients with recurrence of disease within the first year post transplant; patients with no disease recurrence within the same time frame, and healthy control population. Maximal potential level of TGF- β production are indicated: ■ - High ; □ - Intermediate ; ▒ - Low. The potential to produce high levels of TGF- β is significantly more prevalent among patients with no recurrence of hepatitis C compared with LTx recipient with recurrence ($P=0.042$).

was apparent among patients resistant to disease recurrence, while only 50% of patients with recurrent hepatitis C carried this haplotype ($P=0.042$, Figure 1). Patients with recurrence of disease showed phenotypic distribution similar to healthy control population.

Figure 2 represent the phenotypic profile of IL-10 production in hepatitis patients and controls. While the majority (87.5%) of patients without recurrence of hepatitis C are low producers of IL-10, less than half of patients with recurrence of disease displayed this phenotype ($P=0.07$). Interestingly, among healthy controls, an even lower percentage carry this haplotype, and the most prevalent profile is of intermediate producers.

The distribution of IFN- γ phenotypes is quite comparable between patients with disease recurrence and controls, and not significantly different from the observed profile in patients with no HCV recurrence. Nevertheless, a tendency towards higher production of IFN- γ was noted among LTx recipients with no recurrence (Fig. 3; $P=0.1$).

DISCUSSION

The role of cytokines as mediators of post transplant events has been intensely investigated over the past few

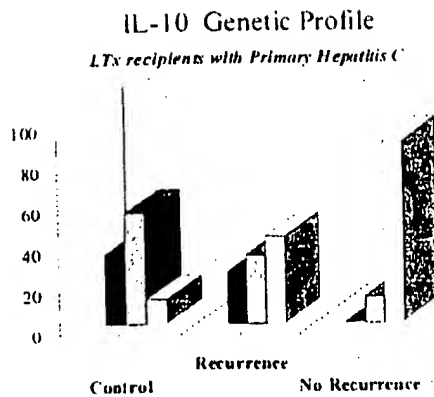


FIGURE 2. IL-10 production profile in LTx recipients with primary hepatitis C. The three study groups include patients with recurrence of disease within the first year post transplant; patients with no disease recurrence within the same time frame, and healthy control population. Low producers of IL-10 are more frequent among LTx recipients with no hepatitis C recurrence ($P=0.07$). Maximal potential level of IL-10 production are indicated: ■ - High; □ - Intermediate; □ - Low.

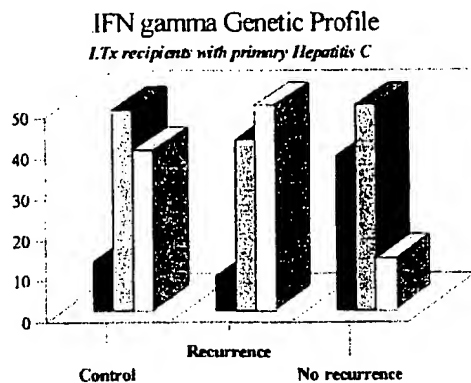


FIGURE 3. IFN- γ phenotypes in LTx recipients with primary hepatitis C. The over all profile is quite comparable between the three study groups. Nevertheless, a tendency towards higher production of IFN- γ was noted among LTx recipients with no recurrence ($P=0.1$). Maximal potential level of IFN- γ production are indicated: ■ - High; □ - Intermediate; □ - Low.

years. Nonetheless, most of the different methods that were employed [ELISA for serum levels of cytokines; RT-PCR or in-situ hybridization for intra-graft measurements etc.] suffer from several disadvantages. For example, measurements of cytokine levels in the serum are not representative of the production and expression levels in a particular organ; Analysis of cytokine data directly from allograft biopsies are informative only if taken at exactly the appropriate timing (20). Consequently, although there is no doubt that cytokines play a major role in allograft rejection/acceptance, currently there are no reliable means to use cytokine information to predict transplantation related complications.

Potential correlations between cytokine gene polymorphism and organ transplantation outcome were analyzed in several studies. The promising data triggered numerous studies in which cytokine gene polymorphism was analyzed

in relation to allograft rejection or other transplantation complications (17-19, 21). Some of these studies, however, disputed earlier findings and revealed many controversies (22, 23).

Our study was designed to analyze potential association between cytokine gene polymorphism in liver allograft recipients and two immunological phenomena: graft rejection/acceptance and protection against recurrence of hepatitis C. While no significant differences in genetic cytokine profile were detected between patients with and without rejection episodes, several deviations from the control group profile were noted, that deserve further investigation.

The genetic ability to produce only low levels of IL-10 was significantly more prominent in LTx recipients with early rejection episodes as compared with healthy controls ($P=0.042$). Although the difference between patients who rejected and those who did not reject did not reach statistical significance, there is a definite trend indicating that more of the non-rejectors have the genetic ability to produce high levels of IL-10, whereas the majority of rejectors can produce only low levels of IL-10. This pattern is in agreement with previous reports correlating the IL-10 low-producer genotype with susceptibility to cardiac allograft (17) and to renal allograft rejections (21). Recently, Shinozaki et al. (24) demonstrated that recombinant human IL-10, when transduced into rat hepatic grafts, significantly prolonged median graft survival ($P=0.0021$). It is well established that IL-10 is a potent regulator, decreasing immunoproliferative and inflammatory responses. IL-10 can directly suppress T cell proliferation, specifically inhibiting their IL-2 production (25, 26). Additionally, IL-10 can indirectly prevent antigen-specific T cell proliferation by down-regulation of MHC class II expression on monocytes, leading to reduced ability to present antigens (27). It is therefore reasonable to assume that recipients with the ability to produce only low levels of IL-10 are more prone to rejection episodes.

Our data further indicate that among LTx recipients who did not reject within the first 4 weeks, the ability to produce low levels of IL-6 was more prevalent than in healthy controls ($P=0.06$). Interestingly, the frequency of IL-6 low production phenotype in our healthy population was only 2% whereas in a study by Fishman et al. (10), that homozygous C/C haplotype was present in 18% of 383 healthy individuals. Similarly, the allelic frequency of the C allele in that study was reported at 40.3% and in the current control population it was only 25.5%. These observations raise a question regarding the normal distribution of cytokine alleles in different ethnic and racial populations. The allelic variant of IL-6 encoding for C in position -174 of the promoter was found to be associated with significantly lower levels of plasma IL-6 in-vivo. Additionally, it was shown that cells carrying this genotype are less amenable to LPS or IL-1 stimulation (10). It is therefore possible that cells with low levels of IL-6 are also less responsive to allostimulation, and hence, less susceptible to rejection episodes. A larger cohort of LTx recipient should be analyzed in order to substantiate this hypothesis.

Recently, Bathgate et al. (28) reported that patients that are homozygous for TNF- α A/A genotype at position -308 of the promoter region are more likely to suffer from acute cellular rejection after liver transplantation. No such correlation was noted in our patient cohort. Moreover, neither of our patients, nor any of the control samples carry this par-

ticular genotype, whereas 19/144 (>13%) of patients in Bathgate's study were A/A positive. In view of the genetic location of TNF- α within the MHC region, and the known ethnic and racial genetic make-up of this system, one may speculate that the distribution of the TNF alleles is also population dependent. Our findings are in apparent disagreement also with other aspects of Bathgate's results—mainly with regard to IL-10 potential association with rejection status. Nevertheless, on a closer examination it is apparent that our analysis of IL-10 promoter focused on three different polymorphic sites while the other study analyzed polymorphism only at position -1082. Finally, our definition of rejection was strictly limited to early cellular rejection episodes occurring within the first 6 weeks post transplant.

Of particular interest is the role of cytokine polymorphism in recurrence of hepatitis C in LTx recipients. Hohler et al. (15) report on an association of the susceptibility to hepatitis C infection, in general, with polymorphism at the TNF- α promoter. In fact, two separate mutations were analyzed. However, while the polymorphism at position -238 is associated with infection status ($P < 0.009$), no such correlation was found with a different mutation at position -308. Similarly, our data—analyzing TNF- α genes for polymorphism at position -308—showed no statistical significant difference among patients with or without recurrence of disease within the first year post transplant. A different approach was used by Rosen et al. (29) who studied polymorphism in donor derived cells and their role in determining severity of hepatitis C recurrence in LTx recipients. In this study, yet again, polymorphism at position -238 of the TNF- α promoter was identified as having significant correlation.

In our study, the vast majority (87.5%) of non-recurrent patients were typed as low producers of IL-10, whereas only 42% ($P = 0.07$) and 12% ($P < 0.0001$) of recurrent patients and healthy control, respectively, carried that genotype. Furthermore, a tendency toward high production of IFN- γ in non-recurrent LTx recipient was noted compared with recurrent patients. That the genetic ability to produce low levels of IL-10 is more prevalent in non-recurrent patient is not surprising. IL-10 down regulates MHC expression on antigen presenting cells (26). Therefore, patients with low secretion of IL-10 should have better capabilities to present antigens (i.e., viral peptides) in the context of MHC molecules, leading to improved elimination of hepatitis infection. Indeed, Edwards-Smith et al. (30) reported that hepatitis C patients, genotyped as high IL-10 producers, have poor response to IFN- α therapy. One may also speculate that low IL-10 production can skew the immune system into the Th1 type of response, which again will facilitate the clearance of viral load.

Although not statistically significant, the percentage of non-recurrent patients having the genetic ability to produce high levels of IFN- γ in our study was 3–4 times greater than in recurrent patients. Two immunological rationales argue in favor of these findings: IFN- γ role as a Th1 cytokine; and its ability to increase the processing and presentation of viral antigens by MHC class I molecules. Indeed, serum specimens from hepatitis C infected patients contain significantly lower levels of soluble IFN- γ as compared with controls (31). Additionally, it has been shown that IFN- γ can inhibit the replication of viruses in infected cells, directly reducing viral load (32, 33).

The most intriguing observation was that all (100%) of patients with no recurrence of hepatitis C carried the high producer phenotype for TGF- β ($P = 0.042$ compared with recurrent patients). TGF- β is a profibrinogenic cytokine known to be involved in development of fibrosis in transplanted organs (19) as well as in hepatocytes of patients with chronic viral hepatitis (34, 35). TGF- β is also known to be involved in suppression of immune responses and in down-regulation of growth promoting activities. Therefore, the finding that all non-recurrent hepatitis C LTx recipients carry a promoter polymorphism predisposing them to high production of TGF- β was unexpected and quite puzzling. Nonetheless, supportive data was obtained by McKeir et al. (36), who reported that TGF- β may down-regulate HIV-1 viral replication in infected myeloid cells in-vitro. These observations imply a dual function for TGF- β as was indeed shown by Erard et al. (37) in a recent publication. Thus, while TGF- β and IL-4 individually suppress immune responses, when presented simultaneously they can cooperate to regulate the differentiation of CD8 cells toward the type 1 response. Namely, addition of both TGF- β and IL-4 was reported to induce IFN- γ secretion by CD8 positive cell cultures and enhance CD8 cytotoxicity. Further studies should address the true nature of TGF- β effects with regard to hepatitis C infection and elimination.

In summary, the multifactorial nature of post transplant immune responses, especially in the early post LTx period, complicate our ability to differentiate the role and contribution of individual parameters for the development of rejection. Our results demonstrate an additional factor that should be considered—the genetic make-up of the studied population. We therefore propose that only a prospective, well-controlled analysis or a larger cohort of patients can decipher the true role of cytokine gene polymorphism in LTx rejection. Nonetheless, our data strongly suggest an association between cytokine gene polymorphism of TGF- β , IL-10 and IFN- γ and recurrence of hepatitis C. Corroboration of our finding may point towards new therapeutic strategies.

REFERENCES

1. Rubinstein M, Dinarello CA, Oppenheim JJ, Hertzog P. Recent advances in cytokines, cytokine receptors and signal transduction. *Cytokine Growth Factor Rev* 1998; 9: 175.
2. Mosman TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996; 17: 138.
3. Hassig A, Kremer H, Liang WX, Stampfli K. The role of the Th-1 to Th-2 shift of the cytokine profiles of CD4 helper cells in the pathogenesis of autoimmune and hypercatabolic diseases. *Med Hypotheses* 1998; 51: 59.
4. Miossec P, van den Berg W. Th1/Th2 cytokine balance in arthritis. *Arthritis Rheum* 1997; 40: 2105.
5. Bidwell JL, Wood NAP, Morse HR, Olomolaiye OO, Laundry GJ. Human cytokine gene nucleotide sequence alignments. *Eur J Immunogenet* 1998; 25: 83.
6. Bidwell JL, Wood NAP, Morse HR, Olomolaiye OO, Laundry GJ. Human cytokine gene nucleotide sequence alignments: supplement 1. *Eur J Immunogenet* 1999; 26: 135.
7. Pravica V, Asderakis C, Perry C, Hajeer A, Sinnott PJ, Hutchinson IV. In vitro production of IFN- γ correlates with CA repeat polymorphism in the human IFN- γ gene. *Eur J Immunogenet* 1999; 26: 1.
8. Wilson AG, Symons JA, McDowell TL, McDevitt HO, Duff GW. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. *Proc Natl Acad Sci USA* 1997; 94: 3195.
9. Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ, Hutchinson IV. An investigation of polymorphism in the interleukin-10 gene promoter. *Eur J Immunogenet* 1997; 24: 1.

10. Fishman D, Faulda G, Jeffery R, et al. The effect of novel polymorphism in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic onset juvenile chronic arthritis. *J Clin Invest* 1998; 102: 1369.
11. He B, Xu C, Yang B, Landtblom AM, Fredrikson S, Hillert J. Linkage and association analysis of genes encoding cytokines and myelin proteins in multiple sclerosis. *J Neuroimmunol* 1998; 86: 13.
12. Mulcahy B, Waldron-Lynch F, McDermott MF, et al. Genetic variability in the tumor necrosis factor-lymphotoxin region influences susceptibility to rheumatoid arthritis. *Am J Hum Genet* 1996; 59: 676.
13. Cabrera M, Show MA, Sharples C, et al. Polymorphism in the tumor necrosis factor genes associated with mucocutaneous leishmaniasis. *J Exp Med* 1995; 182: 1259.
14. Hohler T, Kruger A, Gerken G, et al. A Tumor necrosis factor- α (TNF- α) promoter polymorphism is associated with chronic hepatitis B infection. *Clin Exp Immunol* 1998; 111: 579.
15. Hohler T, Kruger A, Gerken G, et al. Tumor necrosis factor α promoter polymorphism at position -238 is associated with chronic active hepatitis C infection. *J Med Virol* 1998; 54: 173.
16. Halder NA, McLaren A, Marshall SE, Morris PJ, Welsh KI. Is renal graft survival predetermined by the recipient's cytokine profile? *Transpl Proc* 1999; 31: 289.
17. Turner D, Simon CD, Yonan N, et al. Cytokine gene polymorphism and heart transplant rejection. *Transplantation* 1997; 64: 776.
18. Awad M, Pravica V, Perrey C, et al. CA repeat allele polymorphism in the first intron of the human interferon-gamma gene is associated with lung allograft fibrosis. *Human Immunol* 1999; 60: 343.
19. Awad MR, El-Gamel A, Hasleton O, Turner DM, Sinnott PJ, Hutchinson IV. Genotypic variation in the transforming growth factor-beta 1 gene: association with transforming growth factor, beta 1 production, fibrotic lung disease, and graft fibrosis after lung transplantation. *Transplantation* 1998; 66: 1014.
20. Nathan C, Sporn M. Cytokines in context. *J Cell Biol* 1991; 113: 981.
21. Sankaran D, Asderakis A, Ashraf S, et al. Cytokine gene polymorphisms predict acute graft rejection following renal transplantation. *Kidney Int* 1999; 56: 281.
22. Densen CG, Hutchinson IV, Cooper A, Yonan N, Brooks NH. Effect of interleukin-10 polymorphism in cardiac transplant vasculopathy. *JHLT* 2000; 19: 98.
23. Zeevi A, Hutchinson I, Pravica V, et al. The impact of cytokine gene polymorphism on pediatric heart allograft outcome. *JHLT* 2000; 19: 71.
24. Shinozaki K, Yahata H, Sakaguchi T, Ito H, Dohi K. Allograft transduction of IL-10 prolongs survival following orthotopic liver transplantation. *Gene Ther* 1999; 6: 816.
25. de Waal MR, Yssel H, de Vries JE. Direct effect of IL-10 on a subset of human CD4⁺ T-cells clones and T cells: specific inhibition of IL-2 production and proliferation. *J Immunol* 1993; 150: 4754.
26. Zeller JC, Panoskalis-Mortari A, Murphy WJ, et al. Induction of CD4⁺ T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF- β . *J Immunol* 1999; 163: 3684.
27. de Waal Malefyt R, Haanen J, Spits H, et al. Interleukin-10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via down-regulation of class II major histocompatibility complex expression. *J Exp Med* 1991; 174: 915.
28. Bathgate AJ, Pravica V, Perry C, et al. The effect of polymorphism in tumor necrosis factor- α , interleukin-10 and transforming growth factor- β 1 genes in acute hepatic allograft rejection. *Transplantation* 2000; 69: 1514.
29. Rosen HR, Lantz JJ, Rose SL, et al. Donor polymorphism of tumor necrosis factor gene: relationship with variable severity of hepatitis C recurrence after liver transplantation. *Transplantation* 1999; 68: 1898.
30. Edwards-Smith CJ, Jonsson JR, Purdie DM, Banal A, Shorthouse C, Powell EE. Interleukin-10 promoter polymorphism predicts initial response of chronic hepatitis C to interferon α . *Hepatology* 1999; 30: 526.
31. Cribier B, Schmitt C, Rey D, Lang JM, Krin A, Stoll-keller F. Production of cytokines in patients infected by hepatitis C virus. *J Med Virol* 1998; 55: 89.
32. Ramsay AJ, Rudy J, Ramshaw IA. A case for cytokines as effector molecules in the resolution of virus infection. *Immunol Today* 1993; 14: 155.
33. Romero R, Lavine E. Cytokine inhibition of the hepatitis B virus core promoter. *Hepatology* 1996; 23: 17.
34. Murawaki Y, Nishimura Y, Ikuta Y, Idobe Y, Kitamura Y, Kawasaki H. Plasma transforming growth factor-beta 1 concentrations in patients with chronic viral hepatitis. *J Gastroenterol Hepatol* 1998; 13: 680.
35. Tsushima H, Kawata S, Tamura S, et al. Reduced plasma transforming growth factor-beta 1 levels in patients with chronic hepatitis C after interferon- α therapy: association with regression of hepatic fibrosis. *J Hepatology* 1999; 31: 1.
36. McKeir V, Gu Z, Wainberg MA, Hiscott J. Inhibition of human immunodeficiency virus type I multiplication by transforming growth factor beta 1 and AZT in HIV-1-infected myeloid cells. *J Inter Cytokine Res* 1995; 15: 849.
37. Erard F, Garcia-sanz JA, Moriggl R, Wild M-T. Presence or absence of TGF- β determines IL-4-induced generation of type 1 or type 2 CD8 T cell subsets. *J Immunol* 1999; 162: 209.

Received July 17, 2000.

Accepted October 10, 2000.